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Abstract

The steps of two immunofluorescent-antibody-based detection methods were evaluated for their efficiencies in detecting Giardia cysts and Cryptosporidium oocysts. The two methods evaluated were the American Society for Testing and Materials proposed test method for Giardia cysts and Cryptosporidium oocysts in low-turbidity water and a procedure employing sampling by membrane filtration, Percoll-Percoll step gradient, and immunofluorescent staining. The membrane filter sampling method was characterized by higher recovery rates in all three types of waters tested: raw surface water, partially treated water from a flocculation basin, and filtered water. Cyst and oocyst recovery efficiencies decreased with increasing water turbidity regardless of the method used. Recoveries of seeded Giardia cysts exceeded those of Cryptosporidium oocysts in all types of water sampled. The sampling step in both methods resulted in the highest loss of seeded cysts and oocysts. Furthermore, much higher recovery efficiencies were obtained when the flotation step was avoided. The membrane filter method, using smaller tubes for flotation, was less time-consuming and cheaper. A serious disadvantage of this method was the lack of confirmation of presumptive cysts and oocysts, leaving the potential for false-positive Giardia and Cryptosporidium counts when cross-reacting algae are present in water samples.

Keywords

methods, detection, comparison, cryptosporidium, giardia, two, water

Disciplines

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Comparison of Two Methods for Detection of *Giardia* Cysts and *Cryptosporidium* Oocysts in Water

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The steps of two immunofluorescent-antibody-based detection methods were evaluated for their efficiencies in detecting *Giardia* cysts and *Cryptosporidium* oocysts. The two methods evaluated were the American Society for Testing and Materials proposed test method for *Giardia* cysts and *Cryptosporidium* oocysts in low-turbidity water and a procedure employing sampling by membrane filtration, Percoll-Percoll step gradient, and immunofluorescent staining. The membrane filter sampling method was characterized by higher recovery rates in all three types of waters tested: raw surface water, partially treated water from a flocculation basin, and filtered water. Cyst and oocyst recovery efficiencies decreased with increasing water turbidity regardless of the method used. Recoveries of seeded *Giardia* cysts exceeded those of *Cryptosporidium* oocysts in all types of water sampled. The sampling step in both methods resulted in the highest loss of seeded cysts and oocysts. Furthermore, much higher recovery efficiencies were obtained when the flotation step was avoided. The membrane filter method, using smaller tubes for flotation, was less time-consuming and cheaper. A serious disadvantage of this method was the lack of confirmation of presumptive cysts and oocysts, leaving the potential for false-positive *Giardia* and *Cryptosporidium* counts when cross-reacting algae are present in water samples.

Accurate evaluation of *Giardia* and *Cryptosporidium* removal in water treatment processes requires a reliable method for measuring the concentrations of these pathogens in water. As stated in the 18th edition of *Standard Methods for the Examination of Water and Wastewater* (1): "Although methods for detecting cysts in water have been available since 1975, no comparative studies of method efficiency, precision or sensitivity have been reported with a variety of waters under different conditions." The objective of the work reported here was to evaluate and select methods capable of measuring the concentrations of *Giardia* cysts and *Cryptosporidium* oocysts that would be accurate, reliable, flexible, and verifiable. The ultimate goal was to use the best method to measure the performance of various water treatment processes in studies reported elsewhere (7).

Two methods for detecting *Giardia* cysts and *Cryptosporidium* oocysts in water were evaluated. Both methods follow flotation steps and immunofluorescence staining originally described by Riggs et al. (9) and proposed by Sauch (11). The methods were (i) the American Society for Testing and Materials (ASTM) method (2), often referred to as the EPA (Environmental Protection Agency) or the yarn-wound method; and (ii) an alternate method, referred to as such and applied principally by Ongerth and coworkers (4, 8). Details of these methods are summarized in Table 1.

The ASTM method involves sampling 100 liters (or more) of water through a 1.0-μm-porosity polypropylene yarn cartridge filter, extracting the particulates from the cartridge filter by cutting it apart and washing the fibers, and concentrating the extracted particulates by centrifugation. The concentrated particulates are then processed to selectively concentrate cysts and oocysts by flotation in 50-ml tubes on a Percoll-sucrose gradient. The particulates recovered at the interface of the Percoll-sucrose gradient are stained with fluorescently tagged antibodies on 25-mm-diameter, 0.2-μm-pore-size cellulose acetate filters. After being mounted on slides, the membrane filters are scanned with a UV epifluorescent microscope for objects of the right size, shape, and fluorescence characteristic of *Giardia* cysts and *Cryptosporidium* oocysts. On finding such objects, the microscope optics are switched to phase contrast to look for internal morphological characteristics inside the detected organisms. Organisms determined to meet the fluorescence detection criteria are counted as either presumptive *Giardia* cysts or *Cryptosporidium* oocysts. Organisms with the right fluorescence characteristics and shown to have the respective internal morphological characteristics are counted as either confirmed *Giardia* cysts or *Cryptosporidium* oocysts.

The alternate method involves filtration of a 40-liter water sample through either a 293- or a 142-mm-diameter, 2.0-μm-pore-size polycarbonate membrane filter, recovery of particles from the filter by rinsing and squeegeeing them from the surface, and concentration of the particulates by centrifugation. The cysts and oocysts are then selectively concentrated from other particulates by flotation in 15-ml tubes on a two-step Percoll-Percoll gradient, followed by immunofluorescent-antibody (IFA) staining on 13-mm-diameter, 2.0-μm-pore-size polycarbonate membrane filters. After being mounted on slides, the membrane filters are scanned with a UV epifluorescent microscope for objects of the right size, shape, and fluorescence characteristic of *Giardia* cysts and *Cryptosporidium* oocysts. Confirmation of internal structures is not done in this method.

MATERIALS AND METHODS

Approach. The approach used in the study was to evaluate all steps involved in detection of the organisms: sampling, concentration, flotation, IFA staining,

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TABLE 1. Summary of two methods for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water

Method	Sampling	Concn	Purification	IFA assay	Enumeration
ASTM	Filter 380-liter sample through a 10-in- (254-cm)-long, 1.0-μm-nominal-pore-size polypropylene yarn-wound cartridge filter. Process within 48 h.	Section filter fibers with scalpel. Elute in 3–4 liters of eluting solution on shaker platform or in mechanical stomacher. Sonicate final 1-liter eluate concentrate for 5 min in water bath sonicator. Centrifuge at 1,050 × *g* for 10 min at 4°C in large-vol acute-bottom centrifuge tubes in swinging-bucket rotor. Record packed pellet vol. Aspirate supernatant; resuspend pellet with equal vol of 10% formalin. Store at 5°C.	Vortex up to 1-ml packed pellet in 20-ml vol of eluting solution in 50-ml centrifuge tube. Underlay with 30 ml of Percoll-sucrose gradient (specific gravity, 1.10). Centrifuge at 1,050 × *g* for 10 min at 4°C in swinging bucket rotor, no brake, with slow acceleration and deceleration. Aspirate top 20 ml plus interface and 5 ml below interface; transfer to fresh 50-ml tube. Dilute to 50 ml in eluting solution; centrifuge for 10 min at 1,050 × *g*. Aspirate to 5 ml of concentrate.	Assess particle density of a sample. Determine dilution necessary for staining on 25-mm filters. Dilute sample to 1 ml, or apply 1 ml directly to 25-mm, 0.2-μm-pore-size cellulose acetate membrane filter, placed on a filtration manifold set at 50–100 mm Hg (7–13 kPa). Rinse each filter with 2 ml of 1% BSA. Apply 0.5 ml of diluted primary antibody. Incubate for 25 min at room temp. Rinse 5 times with 2 ml of PBS. Apply 0.5 ml of diluted labeling reagent. Incubate for 25 min at room temp; shield from light. Rinse 5 times with 2 ml of PBS. Apply ethanol dehydration series, 10–95% for phase confirmation. Mount slides with DABCO-glycerol. Incubate at 37°C with 20 min of clearing.	Examine slides under ×200–400 epifluorescent microscope. Count presumptive cysts: bright green fluorescent, 8–16-μm ovals for *Giardia* sp. and 2–6-μm spheres for *Cryptosporidium* sp. confirm presumptive counts under phase or differential interference contrast optics, checking internal morphology.
Alternate	Filter through 293- or 142-mm, 1.0- or 2.0-μm-pore-size polycarbonate membrane filter. Vacuum pressure at 200–250 mm Hg (27–33 kPa).	Place filters on a slanting shield. Rinse filter 3 times with filtered (0.2-μm pore size) water or 0.01% Tween 80 to a vol of about 200 ml. Collect rinsings in 50-ml tubes. Centrifuge at 750 × *g* for 15 min at 10°C. Aspirate down to 5 or 10 ml. Add 2% formalin to concentrate. Store at 5°C.	Prepare solutions of PBS with Percoll, one at 1.05 and one at 1.09 specific gravity. Pipette 4 ml of 1.09-specific-gravity layer into 15-ml centrifuge tubes; overlay with 4 ml of the 1.05-specific-gravity solution. Overlay 1–2-ml sample concentrate on top. Centrifuge for 35 min at 10°C and 750 × *g* in swinging-bucket rotor, with acceleration for 6 min and deceleration for 9 min.	Pipette entire vol of gradient above the pellet. Apply directly to 13-mm luer-lock syringe attached to 13-mm filter cartridge, with 13-mm, 2.0-μm-pore size polycarbonate membrane filters inside. Apply sample with syringe. Seal outlet with Parafilm. Apply 300 μl of 1% BSA in PBS. Incubate with 1% BSA at 37°C for 30 min. Stain as in ASTM method. Mount coverslips with elvanol-DABCO-glycerol.	Examine slides under ×200–400 epifluorescent microscope. Count presumptive cysts: bright green fluorescent, 8–16-μm ovals for *Giardia* sp. and 2–6-μm spheres for *Cryptosporidium* sp.

and enumeration. The ultimate goal was to evaluate and compare two detection methods, using three criteria for comparison.

First, the applicability to cyst and oocyst seeding experiments in full- or pilot-scale water treatment plants was evaluated. This was done by spiking water samples with 1,000 *Giardia* cysts and 1,000 *Cryptosporidium* oocysts and determining the number of organisms recovered by the two methods. A higher percent recovery indicated a greater method sensitivity, and thus, a smaller sample volume was required to achieve the same limit of detection.

The second factor in method comparison was the applicability of the methods to detection of *Giardia* cysts and *Cryptosporidium* oocysts in environmental water samples of varying water quality (turbidity and algal content). This was determined by spiking cysts and oocysts into water samples of different quality: untreated surface water of turbidity ranging between 1 and 30 nephelometric turbidity units (NTU), partially treated (flocculated and unsettled) water, and treated water from plant filter effluent. Cyst and oocyst recoveries by the two methods were compared in three types of water.

The third criterion used in method evaluation was the economics associated with the two methods, which was evaluated on the bases of equipment and reagent costs, amount of time required to complete the analysis, and overall complexity of the methods.

Organisms. Heat-inactivated and formalin-fixed *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts were obtained from BioVir (Benecia, Calif.). *Giardia* cysts and *Cryptosporidium* oocysts were seeded into a large water container at known concentrations. Organism numbers were established by counting on a hemocytometer.

ASTM method. (i) Sampling. A 16-liter volume of water containing specific numbers of *Giardia* cysts and *Cryptosporidium* oocysts was placed in a 20-liter container. Sampling was performed in accordance with ASTM Standard Method 9711 (1) by filtering the spiked water through an AMF Cuno Microwynd II polypropylene yarn filter with rubber washers inserted at each filter end inside the plastic cartridge to ensure that leakage around the filter ends did not occur.

(ii) Extraction and concentration. Filters were removed from the cartridge, cut off the support core, divided into three aliquots, and washed in 2-liter beakers each containing 1 liter of eluting solution on a rotating shaker platform. The eluting solution consisted of phosphate-buffered saline (PBS), 1% Tween 80, and 1% sodium dodecyl sulfate. The 3- to 4-liter volume of the eluate was concentrated by repeated centrifugation first in 1-liter flat-bottomed polypropylene centrifuge bottles, then in 175-ml conical centrifuge tubes, and finally in 50-ml conical centrifuge tubes, resulting in a final concentrate volume of about 5 ml, including the settled pellet. The sample concentrates were preserved in an equal volume of 3.7% formalin and refrigerated until flotation on Percoll-sucrose gradients.

(iii) Flotation. One to 20 dilutions of the eluted particulates were prepared with eluting solution in 50-ml conical centrifuge tubes which were sonicated in a water bath sonicator for 10 min. After vigorous vortexing, the 20-ml particulate suspension was underlaid with 30 ml of Percoll-sucrose mixture (specific gravity, 1.10), using a 14-gauge, 10-cm-long, stainless-steel cannula attached to a 50-ml syringe. The Percoll-sucrose gradients were centrifuged at 10°C in a swinging-bucket rotor, at 1,050 × *g*, for 35 min, including 6 min of acceleration and 9 min of deceleration. The gradient interface was clearly visible for harvesting from 5 ml above, through, and 5 ml below the interface. To avoid losses that might occur by aspirating off the 20-ml sample layer, the entire 20-ml sample layer, the interface, and 5 to 10 ml below the interface were harvested from the gradient with a Pasteur pipette. Each interface harvest was transferred to a fresh 50-ml tube, and the volumes were adjusted to 50 ml with additional eluting solution; the harvests were pelleted by centrifugation at 10°C and 1,050 × *g* in a swinging-bucket rotor for 10 min of maximum acceleration with no brake. Supernatants were aspirated off, leaving a 5-ml concentrated sample volume, including the pellet.

(iv) Indirect fluorescent staining. Twenty-five-mm diameter, 0.2-μm-pore-size cellulose acetate membrane filters, which were wetted in PBS, were placed onto a 10-well (25-mm diameter) Hoefer manifold. A continual vacuum of 51 to 102 mm Hg (ca. 7 to 14 kPA) was applied to the manifold to seat the filters in place and allow drainage as needed. Stainless-steel well weights then were placed on top of each filter. Two milliliters of 1% bovine serum albumin (BSA) was applied to each filter and filters were drained by vacuum. When needed, the sample suspension, which was assessed for particulate density on the microscope, was diluted to produce a monolayer. Five milliliters of each diluted or undiluted sample concentrate from the Percoll-sucrose flotation was applied directly onto each membrane and membranes were drained by vacuum. Wells were rinsed with 2 ml of 1% BSA following application of the samples. The filters were stained with the Hydrofluor Combo (Meridian Diagnostics, Cincinnati, Ohio) kit, which allows simultaneous detection of both *Giardia* cysts and *Cryptosporidium* oocysts. First, 0.5 ml of the primary antibody mixture was applied to each filter and filters were incubated for 30 min at room temperature. After five rinses with 2 ml of PBS, 0.5 ml of fluorescein isothiocyanate labeling reagent was applied. The stainless-steel wells were covered with Parafilm and foil to prevent dehydration of this reagent and to protect it from light. Incubation with this reagent was for 30 min at room temperature and was followed by five rinses with PBS. Next, the filters were treated sequentially with 1 ml each of 10, 20, 40, 80, and 95% ethanol solutions containing 5% glycerol. The cellulose acetate material is ethanol compatible and becomes transparent following dehydration. Seventy-five microliters of 1,4-diazabicyclo-(2.2.2.) octane (DABCO)-glycerol mounting medium was placed on each glass slide needed for filter mounting. The slides were prewarmed to 37°C in an incubator for 20 min. The sample filters were removed from each well of the manifold with forceps and placed on the warmed mounting medium on the glass slide, which was incubated for 15 to 20 min at 37°C in an incubator. The membrane filters, which cleared during the 37°C incubation, were mounted by adding 25 μl of mounting medium and a 25-mm^2 coverslip to the top of each membrane. Then the coverslip on each slide was sealed to the slide with clear fingernail polish.

(v) Enumeration. Each slide was scanned completely for cyst- and oocyst-like objects of the right size, shape, and fluorescence under ×200 to ×400 total magnification, using an epifluorescent microscope. Organisms meeting these criteria were labelled as either presumptive *Giardia* cysts or presumptive *Cryptosporidium* oocysts. The microscope was switched from epifluorescence to phase-contrast microscopy to examine each of the presumptive organisms for internal morphological characteristics. The morphological characteristics identifiable in *Giardia* cysts are nuclei, axonemes, and median bodies. Identification of two of the three morphological characteristics in presumptive *Giardia* cysts allowed them to be categorized as confirmed *Giardia* cysts. Identification of one or more sporozoites inside presumptive *Cryptosporidium* oocysts allowed them to be categorized as confirmed *Cryptosporidium* oocysts. Confirmation was required for the examination of environmental samples containing algae, which in some cases resemble the cysts and oocysts in size, shape, and fluorescence due to antibody cross-reactions.

Alternate method. (i) Sampling. Samples were filtered by vacuum through 2.0-μm-pore-size polycarbonate membrane filters at pressures of 250 to 270 mm Hg (ca. 33 to 36 kPa). The 293-mm-diameter filters in a stainless-steel apparatus were used for samples of more than 20 liters. Smaller-volume samples were filtered through 142-mm-diameter filters with a plastic filter apparatus. The filter inlet tubing was autoclaved. Filter holders were washed with hot soapy water and rinsed with distilled water after each use.

(ii) Extraction and concentration. After filtration, the membranes were removed from the holder and placed on an inclined Plexiglas plate; they were alternately rinsed and squeegeed three times with filtered (0.2-μm pore size) water containing 0.01% Tween 80. The particle eluate was collected in a plastic collection dish, transferred to 50-ml conical tubes, and centrifuged for 15 min at 750 × *g* at 10°C. After aspiration of the supernatant, the remaining liquid and pellet were preserved in 2% formalin.

(iii) Flotation. A Percoll-Percoll step gradient was used to separate the cysts and oocysts from other particulates. The step gradient was produced in a 15-ml conical centrifuge tube, using 4 ml of 1.09-specific-gravity Percoll overlaid with 4 ml of 1.05-specific-gravity Percoll. A particle eluate volume of 2 ml was applied over the 1.05-specific-gravity Percoll layer. Then the tube was centrifuged at 750 × *g* for 35 min at 10°C, including 6 min of acceleration and 9 min of deceleration.

(iv) Indirect fluorescent staining. The entire fluid layer above the pellet in each flotation tube was harvested by pipette and applied directly to a 13-mm-diameter, 2.0-μm-pore-size Nuclepore polycarbonate filter (Livermore, Calif.) housed in an in-line filter holder. The inlet port was connected to a 20-ml syringe. When drainage was not desired, the filter holder outlet port was sealed with Parafilm. Three hundred microliters of 1% BSA in PBS was applied, and filters were allowed to incubate at 37°C for 30 min. Filters were rinsed with 15 ml of PBS and then stained with the Hydrofluor Combo kit (Meridian Diagnostics), according to the kit instructions. After staining, the filters were rinsed with PBS, removed from the filter holders, and mounted on a microscope slide by using a drop of elvanol mounting medium (5), which contained 26.1 mg of DABCO per ml to protect the fluorescence from fading.

(v) Enumeration. Each slide was scanned for cyst- and oocyst-like objects of the right size, shape, and fluorescence under ×200 to ×400 total magnification, using an epifluorescent microscope.

RESULTS

Percent recovery of the two methods in seeded water. The efficiencies of the two methods in detecting seeded *Giardia* cysts and *Cryptosporidium* oocyst are summarized in Table 2. The effectiveness of parasite recovery from spiked water samples depended primarily on the number of analytical steps involved in cyst detection. Both IFA methods were characterized by low recovery efficiency when seeded raw water samples were filtered and then concentrated, transferred to gradients, stained, and enumerated. Higher recovery rates were observed in detecting *Giardia* cysts when the alternate method was employed. Among seeded samples, compared in quadruplicate, an average 49% of the seeded *Giardia* cysts were detected by the alternate method, while the ASTM method was characterized by an average *Giardia* cyst recovery efficiency of 12%.

Losses were demonstrated to occur due to incomplete yarn

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TABLE 2. Average cyst and oocyst recovery from seedings at three points in four replicate raw water samples analyzed by the ASTM method and the alternate method

Point of seeding cysts or oocysts	% Cyst or oocyst recovery (SD)	
	ASTM method	Alternate method
Before sampling and concn		
Giardia sp.	12 (18)	49 (91)
Cryptosporidium sp.	8 (13)	9 (4)
At the gradient		
Giardia sp.	53 (33)	82 (23)
Cryptosporidium sp.	27 (9)	69 (25)
Directly on membranes		
Giardia sp.	72 (14)	86 (16)
Cryptosporidium sp.	56 (8)	78 (9)

cartridge filtration. Up to 5% of *Giardia* cysts and 6% of *Cryptosporidium* oocysts were recovered from the yarn cartridge filtrate by passing it through a 293-mm-diameter, 2.0-μm-pore-size Nuclepore membrane. Recovery rates for *Cryptosporidium* oocysts averaged less than 10% regardless of the method used and, as observed by others (2, 4, 12), depended more on the raw water turbidity and algal content than on the sampling and processing method used for detection.

As shown in Table 2, the sampling step resulted in the highest loss of seeded *Giardia* cysts and *Cryptosporidium* oocysts. When the sampling step was eliminated and cysts were seeded directly onto flotation gradients, the resulting recovery rates increased dramatically. The most appreciable increase in cyst recoveries was observed in analyses for *Cryptosporidium* oocysts. The alternate method, employing Percoll-Percoll flotation, yielded recoveries of over 80% for seeded *Giardia* cysts and *Cryptosporidium* oocysts. An average 53% of *Giardia* cysts and 27% of *Cryptosporidium* oocysts were detected from seeded Percoll-sucrose gradients used in the ASTM method.

When cysts and oocysts were seeded directly onto the membrane filters used for staining, the highest cyst recovery rates resulted. The alternate method was characterized by an average recovery of 86% for *Giardia* cysts and 78% for *Cryptosporidium* oocysts. The elvanol-mounted slides had a relatively dark background, facilitating identification of cysts and oocysts. The ASTM method resulted in recoveries of 72 and 56% for *Giardia* cysts and *Cryptosporidium* oocysts, respectively.

Percent recovery of the two methods in environmental water samples. Testing of parasite recovery efficiencies from raw, flocculated, and filtered water indicated that any increase in water turbidity, whether due to the presence of algae or to that of chemical floc, resulted in a significant decrease in parasite recoveries. Figure 1 presents a summary of parasite recoveries obtained in three types of water: filtered water with a turbidity of 0.5 NTU, raw surface water with a turbidity of 5 NTU, and water from a flocculation basin with a turbidity of 20 NTU. When seeded cysts and oocysts were sampled and processed according to the two methods, the highest recovery rate occurred in filtered water rather than in raw or partially treated water. *Giardia* cysts were recovered at higher rates than were *Cryptosporidium* oocysts, especially when the alternate method was employed. The recoveries resulting from this set of seeding tests were characterized by a high standard deviation, varying from 1 to 90%.

Similar results were obtained when the cysts and oocysts were seeded directly onto flotation gradients or seeded into the staining step. A summary of the results from these trials is presented in Fig. 2. The results indicate that recoveries were consistently higher with the alternate method in all three types of water and that both methods were more effective in detecting seeded *Giardia* cysts than *Cryptosporidium* oocysts.

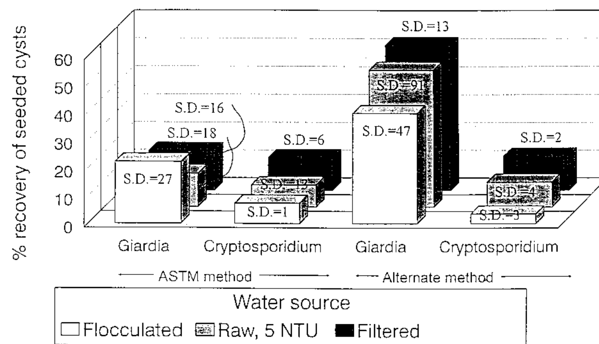

FIG. 1. Water quality versus recovery of *Giardia* cysts and *Cryptosporidium* oocysts seeded before sampling. S.D., standard deviation.

The advantage of sampling by the ASTM method is that much larger volumes of water can be filtered than through the membrane filters used in the alternate method. The use of the 10-well (25-mm diameter) Hoefer manifold for IFA staining on membrane filters was another benefit of using this method. A very significant advantage of the ASTM method over the alternate method was the ability to confirm presumptively counted organisms. The dehydration and clearing of the cellulose acetate membrane filters with ethanol-glycerol permitted examination of internal structures under phase-contrast optics. This is not possible with the polycarbonate membrane used in the alternate method.

A disadvantage of the ASTM method was associated with the use of 25-mm-diameter membrane filters which required a longer time to completely scan and enumerate than the 13-mm-diameter polycarbonate membranes used in the alternate method. Furthermore, background fluorescence was associated with the filter membranes generated by the ASTM method. Experimental modifications made in the ASTM slide preparations (with or without 1% BSA; with or without Evans blue counterstain; with or without the ethanol dehydration series; or with 1, 2, or 3 drops of DABCO-glycerol mounting medium underneath or on top of the filter) did not diminish the green background fluorescence. The membrane background fluorescence made it more difficult to do the scanning

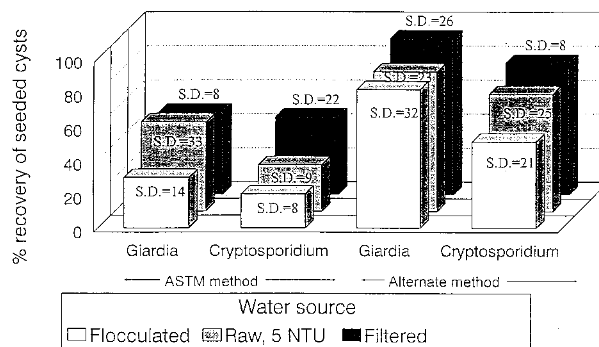

FIG. 2. Water quality versus recovery of *Giardia* cysts and *Cryptosporidium* oocysts seeded on gradients. S.D., standard deviation.

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of the slides for cysts and oocysts, because the contrast between the parasites and the background was diminished. In addition, the cellulose acetate membranes never could be completely flattened. As a consequence, the organisms of interest were found in many different planes of focus.

Elution of parasites and particulates from the membrane filters, employed in the alternate method, was considered easier than elution of the ASTM method's yarn cartridge filters, which required cutting, shaking, and sonication during extraction. The extraction of parasites from polycarbonate membrane filters was found to be more effective when a scraping "squeegee" tool was used. This tool consisted of a paint scraper handle with sections of rubber baseboard lining clipped into the clamp. Losses of *Giardia* cysts and *Cryptosporidium* oocysts were observed during the gradient centrifugation step. These losses were higher in the ASTM method, possibly due to multiple centrifugation steps and transfers employed.

The immunofluorescent staining, according to the alternate method, required manual processing of each of the filters. An advantage of this method was the fact that the elvanol mounting medium in conjunction with the polycarbonate membrane filter consistently produced a black membrane background. This resulted in a sharper contrast between the bright apple-green cysts and oocysts and the membrane. Furthermore, since the membranes were flat, a single plane of focus resulted, making it easier for the analyst to scan and count the parasites. The elvanol mount, due to the resin hardening, also held the coverslip in place, which allowed storage of slides either stacked or on their sides for a period of several months. Moreover, once hardened, the elvanol prevented the cysts and oocysts from migrating across the membrane. This feature permitted easy recounting of slides by one analyst or comparative examination by another analyst.

Economic comparison of the two methods. Economic comparison of the two methods was based on the amount of time needed for sample processing and detection of the parasites, cost of the required equipment, and the number as well as volumes of reagents needed. Manual extraction of parasites from the ASTM yarn sampling filters was found to be a cumbersome and time-consuming procedure. The yarn filter fibers in the sample eluate concentrated with the other particulates from the eluate and were carried through the gradient flotation steps as well. Gradient harvests were too dense to apply to a single 13-mm-diameter filter for staining without dilution in the alternate method, so many more slides were required to examine each sample.

Sampling by the alternate method membrane filtration step required a heavy, expensive, stainless-steel pressure filter apparatus for the 293-mm-diameter filters. Sampling by filtration through yarn cartridge filters, used in the ASTM method, was less costly. However, costs of the nonbreakable centrifuge tubes used in the alternate method were lower than that of the large bottles needed in the ASTM method.

A comparison of the amount of Percoll required during the flotation step in the two methods showed the economic advantage of the Percoll-Percoll gradient over that of Percoll-sucrose. A series of eight Percoll-Percoll gradients, which were centrifuged in the 15-ml centrifuge tubes, required only 42 ml of Percoll. A series of eight of the Percoll-sucrose gradients centrifuged in the 50-ml centrifuge tubes required a total of 145 ml of Percoll. The cost of Percoll is $175.00/liter.

The IFA staining, performed on 13-mm-diameter filters according to the alternate method, required less staining material than that used in staining the 25-mm-diameter filters. The staining of the smaller filters was also less time-consuming.

Preparation of gradients, application of samples, centrifugation time, and the time required to prepare each sample harvested from a gradient for the accompanying immunofluorescent staining procedure accounted for a major block of time in both methods. Actual time spent on the microscope examining the IFA slides was another major factor in evaluation of the methods. The Percoll-Percoll step gradient method and immunofluorescent staining on 13-mm-diameter membranes proved the least time-consuming of the two methods. In a sample seeded with high cyst concentrations, microscopic examination of the 25-mm-diameter filters required 2 h, without confirmation by phase microscopy. A similar sample processed on the 13-mm-diameter slides used in the alternate method required 30 min of microscopy time.

DISCUSSION

Recoveries of *Cryptosporidium* oocysts reported for the ASTM yarn-wound method range from the 70 to 80% (6, 10) to 1% or less (3, 12). *Cryptosporidium* recovery efficiencies reported for the membrane filter sampling range from 10 to 15% (8) to 25 to 35% (4).

The main advantage of the ASTM method was its ability to confirm presumptive cysts and oocysts. The most serious disadvantages of this method were its relatively high cost and the amount of time required to complete it. The alternate method, on the other hand, was found to be less expensive and required less time to complete than the ASTM method. Attractive features of the membrane filter sampling method include the relatively small sample volumes, flexibility and compatibility with frequent seeded controls, and apparently narrow range of performance in comparison to more cumbersome features of the ASTM sampling method. The major limitation of the alternate method was its lack of a confirmation step.

The presence of algae decreased parasite recovery efficiencies more than increases in turbidity caused by inorganic contaminants. Monitoring for concentrations of *Giardia* cysts and *Cryptosporidium* oocysts, naturally occurring in raw or filtered water, requires the use of cellulose acetate membrane filters and the confirmation of internal morphological features by contrast microscopy. As a consequence, because of its ability to distinguish between cross-reacting algal cells and the parasites by contrast microscopy, the ASTM immunofluorescence staining method was considered essential for processing environmental samples. The alternate method has proven more effective in recovering seeded *Giardia* cysts and *Cryptosporidium* oocysts and, therefore, was considered more suitable in parasite seeding experiments. When parasites are seeded at high concentrations, the confirmation step is not critical since the background level of indigenous organisms such as algae is comparatively low.

Experience with detecting *Giardia* cysts and *Cryptosporidium* oocysts and the results from this study indicate that the alternate method, which was more efficient in detecting *Giardia* cysts and *Cryptosporidium* oocysts, be recommended to evaluate water treatment processes that use high concentrations of seeded parasites in which algae, occurring in concentrations much lower than the seeded parasites, are not of concern. The alternate method can be considered for analysis of environmental samples, particularly for low-turbidity waters. When high water turbidity requires higher-volume samples to be collected and examined, and when cross-reacting algae should be differentiated from the organisms of interest, the ASTM method should be used with environmental samples.

The results of this study have indicated that *Giardia* cysts and, especially, *Cryptosporidium* oocysts are lost during the

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gradient flotation steps of both methods. Therefore, it is recommended that the flotation step be avoided when processing treated (filtered) water samples.

A hybrid method, combining the most efficient steps from the two methods, should be investigated. Such a hybrid method should include sampling by membrane filtration only for low-turbidity waters. High-turbidity waters should be sampled by the ASTM cartridge sampling method. Since the Percoll-Percoll step gradient in 15-ml tubes is more economical and had higher parasite recoveries than the Percoll-sucrose gradient, it should be used. Staining on cellulose acetate membranes, as opposed to polycarbonate membranes, allows the demonstration of internal morphological characteristics of the parasites. Consequently, staining on cellulose acetate membranes should be incorporated into a hybrid method. Elvanol mounting medium should not be incorporated into a hybrid method, since as a water-based medium it is not compatible with the dehydrated cellulose acetate membrane and does not allow the membrane to be cleared so that the internal structures of the organisms can be visualized by contrast microscopy.

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